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Mapping Antigenic Sites on the Major Outer Membrane Protein of *Chlamydia trachomatis* with Synthetic Peptides

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The antigenicity of the major outer membrane protein (MOMP) of *Chlamydia trachomatis* was comprehensively evaluated by using overlapping hexapeptide homologs of serovar B MOMP and polyclonal rabbit antisera in a peptide enzyme-linked immunosorbent assay. Of 367 hexapeptides, 152 showed reactivities with at least one antiserum. Seven hexapeptides located within variable domain (VD) IV (residues 288 to 316) were found to be most reactive in terms of their binding titer and frequency, suggesting that VD IV is the immunodominant region within the MOMP as detected by this assay. Peptide-reactive antibodies could also recognize corresponding epitopes on either viable or acetone-permeabilized organisms. The antigenic specificity and immunoaccessibility of epitopes located in VD IV were resolved by absorbing antisera with chlamydial elementary bodies. Six antigenic sites were found in this region and included a B-type-specific site (S1), four subserogroup-specific sites (S2 and S4 to 6), and one species-specific site (S3), each displaying varying degrees of surface exposures on elementary bodies from different *C. trachomatis* serovars.

Chlamydiae are a unique group of obligate intracellular bacteria consisting of three species, *Chlamydia trachomatis*, *Chlamydia psittaci*, and *Chlamydia pneumoniae*. *C. trachomatis* is an important human pathogen causing trachoma and a variety of sexually and perinatally transmitted diseases. *C. trachomatis* is classified into 15 serovars on the basis of antigenic variation in the major outer membrane protein (MOMP). The MOMP is a surface-exposed, integral membrane protein of approximately 40 kilodaltons. It has functional roles for the structural integrity of the extracellular infectious elementary body (EB) and porin properties permitting uptake of essential nutrients such as ATP for the intracellular replicatory form, the reticulate body. Other studies (2, 3, 20, 26, 27, 35, 36, 39) have demonstrated that the MOMP has discrete regions of antigenicity and immunoaccessibility. Comparative DNA analysis from MOMP genes of three different *C. trachomatis* serovars revealed that the MOMP has four regions of amino acid variability interspersed among five regions of amino acid conservation (27). Baehr et al. (2) further suggested that the four regions of variability were surface exposed and the five constant regions were transmembranous. Three of the four variable domains (VDs) have been identified as antigenic sites on the surface of the whole organism, using monoclonal antibodies (MAbs) (2, 27, 29). The MOMP is also an immunoprotective antigen and is the target for neutralizing MAbs. MAbs against MOMPs with a variety of specificities have been found to be protective in in vitro cell culture systems (19, 23, 24), in vivo mouse toxicity (24, 39), and ocular infections in monkeys (39). Since antigenic determinants on this molecule could form the basis for a chlamydial vaccine, determination of the antigenicity, specificity, and immunoaccessibility of MOMP epitopes should permit the rational selection of candidate peptides. In addition, the antigenic sites can be used to study protein structure such as the topography of membrane proteins.

Synthetic peptides have been used to successfully map immunologically important antigenic sites on the MOMP. Stephens et al. (29) used synthetic peptides to map linear epitopes for serovar-specific, subspecies-specific, and species-specific MAbs to VD II and VD IV. Conlan et al. (6) corroborated the findings of Stephens et al. by localizing an L_1 serovar-specific epitope to VD II and subspecies- and species-specific epitopes to VD IV. They further evaluated the fine specificity of MAb binding to substituted peptides modeled after conformationally dependent subspecies epitopes in VD IV (7).

We applied the approach used by Conlan et al. to systematically scan the entire MOMP protein sequence for continuous epitopes capable of binding antibodies elicited by immunizing rabbits with whole, viable EBs of serovar B. Six linear epitopes were located in VD IV. We inferred surface exposure and antigenic specificity of the six immunodominant epitopes by absorption of pooled antisera with whole, native EBs from 12 different *C. trachomatis* serovars.

MATERIALS AND METHODS

The following *C. trachomatis* serovars were used: A (G17/0T), B (TW5/0T), C (TW3/OT), D (UW3/CX), E (UW5/CX), F (UW6/UR), H (UW43/CX), I (UW12/UR), J (UW36/CS), K (UW31/CX), L2 (434/BU), and L3 (404/BU). Strains were grown in HeLa 229 cells, and EBs were purified on Renografin gradients as described elsewhere (23). Portions from each batch of organism used were immunotyped with MAbs to ensure correct serovar specificity (35).

Preparation of polyclonal antisera. Antibodies against viable serovar B EBs were raised in female New Zealand White rabbits (weight, 2 to 2.5 kg) (Roger Tessier, St. Pierre-Jolys, Manitoba, Canada). Each rabbit was intramuscularly immunized with 5×10^8 inclusion-forming units of purified serovar B EBs in 2 ml of phosphate-buffered saline (pH 7.4) emulsified in an equal volume of Freund incomplete adjuvant (Difco Laboratories, Detroit, Mich.). At 21 days later, two intravenous injections of an aqueous EB suspension (10^8 inclusion-forming units each) were given at 7-day intervals. At 10 days after the last intravenous injection, rabbits were bled. The microimmunofluorescence titers of the antisera were 1/2,000 to 1/4,000 with species cross-reactivity, and the

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highest titer in all antisera was observed with the homologous serovar B or B complex serovar EBs.

Peptide synthesis. We used the amino acid sequence of the serovar B MOMP as deduced from the DNA sequence by Stephens et al. (27) and synthesized 367 overlapping hexapeptides covering the entire MOMP B sequence using a commercially available kit (Cambridge Research Biochemicals, Cambridge, United Kingdom) (10). Chemicals used were of analytical reagent grade or the best available grade. The synthesis design and the detailed procedure were supervised by the supplied software and manual. The hexapeptides were synthesized with a Fmoc-chemistry. Successful synthesis was ensured by the simultaneous synthesis of positive and negative control pins and the comparison of the reactivity with test MAbs to pins on which control peptides had been synthesized and supplied by the manufacturer. Three control hexapeptides (ACDEFG, HIKLMN, and PQRSTV) containing difficult coupling residues were subjected to amino acid analysis to evaluate the success of the synthesis. The calculated quantity of peptide on each pin was approximately 250 nmol.

Indirect peptide ELISA. The immobilized peptides were assayed by enzyme-linked immunosorbent assay (ELISA) according to the instructions of the manufacturer, using polyclonal antisera raised against serovar B as first antibodies and goat anti-rabbit immunoglobulins (immunoglobulin A plus immunoglobulin G plus immunoglobulin M) (Cappel 3212-0231) conjugated to horseradish peroxidase as second antibodies at a dilution of 1/1,000. The color was developed with 2,2-azino-bis-(3-ethyl-benzthiazoline-6-sulfonate) (Sigma Chemical Co.) in the dark and read at 405 nm, using a microplate reader (model EL308; Bio-Tek Instruments, Inc.). Antiserum from individual rabbits was used at a dilution of 1/1,000, and pooled antisera were used at a dilution of 1/200. The results were expressed in optical density (OD) values. The solid-phase peptides were reused after the bound antibodies were disocciated by ultrasonification in hot sodium dodecyl sulfate and 2-mercaptoethanol solution.

Absorption of rabbit antisera. To determine the surface exposure and specificity of the antigenic sites on the MOMP as found in native EBs, pooled antisera from seven rabbits (at equal ratios) were preincubated with an excess of viable or acetone-fixed EBs (15 min at room temperature, 10^8 inclusion-forming units per 0.1 ml of antiserum) in phosphate-buffered saline (pH 7.2) containing 2% bovine serum albumin–0.1% Tween at a total volume of 1.2 ml in microcentrifuge tubes at 4°C overnight with continuous rotation. EBs were pelleted at 75,000 × *g*, and the supernatant was assayed in the peptide ELISA, using a 1:200 dilution of the original sera. To exclude the possibility of nonspecific absorption of antibody, we incubated antisera with intact or acetone-fixed HeLa cell debris as a nonrelevant antigen (10^8 cells/0.1 ml of antiserum). Run-to-run experimental variability of antibody binding to specific hexapeptides in terms of OD units was consistently ≤20%. We therefore judged that a ≥50% reduction of OD value was significant after absorption of antisera by EBs when compared with absorption by HeLa cell debris and indicated the presence of that epitope.

RESULTS

Comprehensively scanning the immunogenicity of the MOMP. To analyze the complex polyclonal antibody response to *C. trachomatis* MOMP, we assayed antisera raised by immunizing rabbits with whole serovar B EBs using overlapping hexapeptide homologs of MOMP B sequence in a solid-phase ELISA. Hexapeptide immunoreactivity is shown in Fig. 1A to D. A dramatic variation in the frequency of antibody response to individual hexapeptides among the seven antisera was observed (Fig. 1A). The number of hexapeptides reactive with antibody ranged from 22 in rabbit number 3 to 81 in rabbit number 6. Collectively, 152 of 367 hexapeptides were recognized by at least one antiserum.

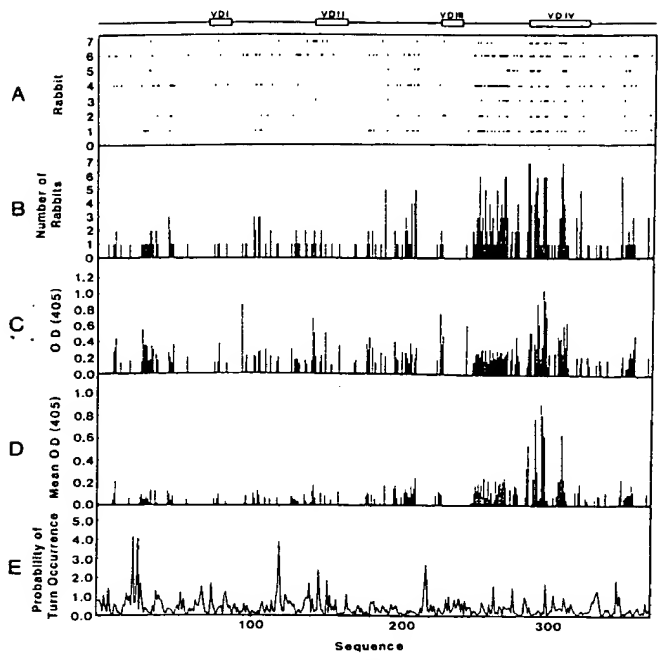

FIG. 1. Profiles of antigenic responses (A to D) and β-turn occurrence of serovar B MOMP (E) as a function of all MOMP B hexapeptides. Antibody recognition of MOMP B is measured by the reactivity of serovar B antibodies to hexapeptide analogs of the MOMP B sequence. Each parameter is plotted as the sequence number corresponding to the first residue of the relevant hexapeptide. (A) The reactivity pattern specific to each of the seven rabbit antisera. Response to any hexapeptide is indicated with a short vertical bar. (B) The frequency of the antigenic response given by the number of rabbit antisera that react with each hexapeptide. (C) The individual titer (as measured by OD unity) or, in cases in which more than one serum reacted, the geometric mean titer of the antisera. (D) The geometric mean OD units to hexapeptide obtained by considering the total number of rabbit antisera investigated. The OD value from a negative reaction antiserum was taken as zero. (E) The MOMP B sequence was analyzed for secondary structure prediction by the Chou-Fasman-based probability of a β-turn occurrence (15).

The titer (as determined by arbitrary OD units at a dilution of 1:1,000) and frequency of reactivity were closely associated. Both the mean frequency and titer of the most reactive peptides were significantly higher than those of the least reactive peptides ($P < 0.05$ and $P < 0.05$, respectively). Seven of the most reactive hexapeptides (hexapeptides 288, 289, 293, 297, 298, 299, and 311 [numbered as first residue of the peptide]) showed high titer (OD value, ≥0.4) and high frequency reactivity (four or more rabbits); 105 infrequently recognized hexapeptides (three or fewer rabbits) had lower titer (OD value, <0.4). Yet exceptions occurred; for instance, 19 hexapeptides showed low titer (OD value, <0.4) but high frequency of reactivity (four or more rabbits), and

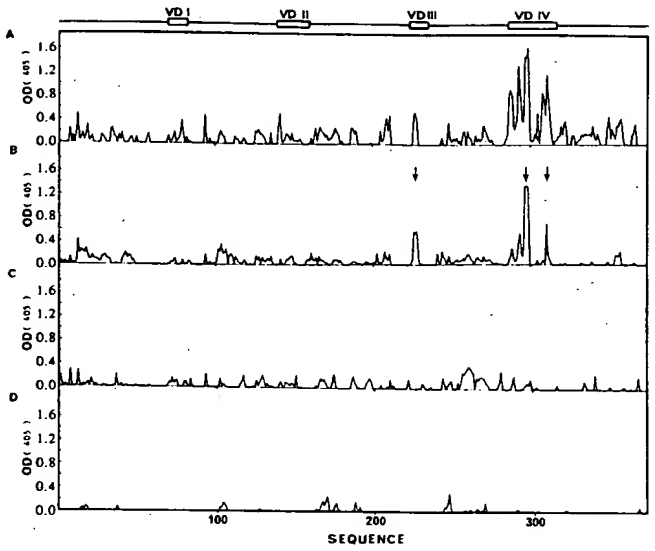

FIG. 2. Characterization of antipeptide antibodies (by arbitrary OD units), using absorption with homologous serovar B EBs of pooled antisera from the seven immunized rabbits. (A) Without absorption; (B) with viable EB absorption (arrows indicate nonabsorbed peaks); (C) with acetone-permeabilized EB absorption; (D) reactivity of a normal rabbit serum with hexapeptides.

21 hexapeptides exhibited high titer (OD value, ≥0.4) but low frequency of reactivity (less than four rabbits).

Hexapeptides which reacted with antibody in both high titer and high frequency can be considered immunodominant. Figure 1D best shows the antigenicity of the B MOMP, since it considers the total number of animals evaluated and the titers obtained from each peptide. The antigenic maxima were centered within VD IV (residues 288 to 316), indicating that VD IV is the most immunogenic region (at least in rabbits) among the entire sequence of the B MOMP. VD II (residues 139 to 160) and VD III (residues 224 to 237) contained peptides which induced high titer antibody but at low frequency; on the other hand, VD I (residues 69 to 83) induced low titer antibody at low frequency. These results indicate that while serovar B MOMP VD I, II, and III are immunogenic, VD IV is immunodominant among immunized rabbits.

We also analyzed the MOMP sequence using several different hydrophilicity scales (14, 16). These analyses did not uncover statistically significant relationships between antigenicity as detected in our assay and estimates of hydrophilicity or surface accessibility (data not shown). A limited correlation was observed between the probability of β-turn occurrence (15) and antigenic regions of the B MOMP (Fig. 1E). Fourteen hexapeptides exhibited a high probability of β-turn occurrence ($P \geq 1.5 \times 10^{-4}$), and five of these (hexapeptides 69, 75, 140, 146, and 299) were located at measurably antigenic peptides ($P < 0.01$).

Determination of surface-exposed epitopes on the B MOMP. We next determined whether peptide-reactive antibodies also recognized surface-exposed epitopes on EBs. The seven antisera were pooled (1:200), and their composite reactivity was assessed in the solid-phase ELISA (Fig. 2A). Both the most reactive and the least reactive peptides showed significant binding to corresponding antibodies, with the highest binding occurring in the immunodominant VD IV region, consistent with the results observed with individual antisera. We preabsorbed antisera with either viable or acetone-fixed EBs from the homologous serovar B. The results obtained after absorption with viable EB showed that most antipeptide antibodies recognized surface-exposed antigenic sites on the MOMP on intact organisms; notable exceptions were antibodies binding to peptides 227, 228, 229, 297, 298, 299, and 311 (Fig. 2B). We also found that nearly all antipeptide antibodies recognized epitopes on acetone-permeabilized organisms (Fig. 2C), indicating that the antibody population detected with the peptide ELISA belongs to those antibodies that can bind to both peptides and whole protein (17, 18). The absorption experiments confirmed the specificity of the antibody-peptide interaction, since acetone-fixed EBs essentially removed all antibodies that we detected as bound to hexapeptides. We also failed to observe a significant binding of serum from an unimmunized rabbit to MOMP hexapeptides (Fig. 2D). We next used the pooled antisera to further analyze epitopes within the immunodominant region VD IV.

High-resolution analysis of epitopes within VD IV. We focused our attention on VD IV because antisera were most reactive to this region. We mapped the antigenic specificity and immunoaccessibility of six epitopes within this region. After absorption of antisera with viable or acetone-fixed EBs from different serovars, we could map six contiguous but discrete linear antigenic sites designated sites 1 to 6 (S1 to S6) from the N-terminal to the C-terminal region of VD IV (Fig. 3).

S1 was defined by antibodies binding to peptide 289, S2 was defined by binding to peptides 293 and 294, S3 was defined by binding to peptides 297 to 299, S4 was defined by binding to peptide 305, S5 was defined by binding to peptides 308 and 309, and S6 was defined by binding to peptides 311 and 312. Four of these epitopes (S1, S2, S3, and S6) were immunodominant (Fig. 1D). Surface-exposed epitopes were assessed by viable EB absorption. S1 exhibited serovar B-type specificity and was surface exposed on serovar B. S2, S4, S5, and S6 showed serogroup specificities and were surface exposed on some but not all serovars. S3 was a species-specific epitope, since it was absorbed by all 12 serovars tested when EBs were permeabilized by acetone and was surface exposed on serovars A, D, E, F, K, L2, and L3 (although with different degrees of accessibility as assessed by a reduction of OD value). S3 was not surface exposed on serovars B, C, H, I, and J. Generally within VD IV, different epitopes on the same serovar or the same epitope on different serovars showed great variations in specificity, immunoaccessibility, and conformational dependency as determined by sensitivity to acetone treatment.

DISCUSSION

Prior successful use of peptide synthesis in studying protein antigenicity (4, 8, 13, 31) and in developing a synthetic subunit vaccine (5, 22, 30, 32) encouraged us to use this approach to resolve *C. trachomatis* MOMP antigenicity and epitope immunoaccessibility. Clearly, synthetic peptides have important limitations in elucidating protein antigenicity. Peptide-based assay systems can only detect those antibodies which can recognize either synthetic peptide epitopes alone or both peptide and native epitopes. Importantly, these antibodies appear to constitute 60 to 70% of an antiprotein antibody population (17, 18). Antibodies detected in our assay fell into this group (Fig. 2). Not evaluated were antibodies which have obligatory conformational re-

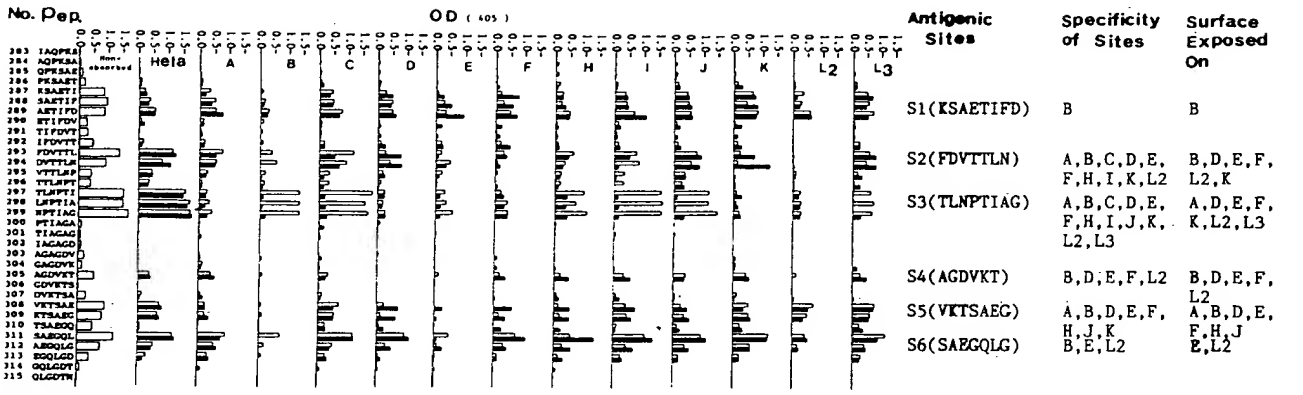

FIG. 3. High resolution characterization of epitopes in VD IV, using nonabsorbed antisera or absorption of pooled antisera with either viable (□) or acetone-permeabilized (■) EBs or HeLa cell debris. The specificities of antigenic sites (S1 to S6) were judged as ≥50% reductions of OD with either viable or fixed EB absorptions compared with absorptions with HeLa cell debris. Surface exposure of the sites was similarly determined with viable EB absorption. Along the vertical axis are the hexapeptide sequences designated by one-letter codes and numbered as the first residue position in MOMP B.

quirements. Our conclusions are thus limited to sequential epitopes mimicked by hexapeptides. This approach can be used as a very convenient scanning strategy to select immunologically relevant peptides on the basis of sequence-specific information (10). Although Conlan et al. (6) used the same approach to map antigenic sites in variable regions of the MOMP, the antigenic structures of the MOMP were not evaluated. Using rabbit polyclonal antisera raised with serovar B EBs, we could comprehensively scan the antigenicity of the entire sequence of the B MOMP, define regions of immunodominance, and infer epitope surface exposure on a variety of chlamydial serovars.

Our observations show that MOMP peptides fall into one of the five following categories according to their reactivities with antibodies: high titer-high frequency, high titer-low frequency, low titer-high frequency, low titer-low frequency, and nonreactive (Fig. 1). Peptides with high frequency and high titer represent the immunodominant sequences in native organisms. The high titer-low frequency binding pattern may be caused by host major histocompatibility complex (MHC) restriction or other factors extrinsic to protein structure and has been seen in other systems, including the malaria vaccine studies (11). Peptides which showed a low frequency-low titer or high frequency-low titer binding pattern may represent immunorecessive regions in the native organisms, or the peptide ELISA system may have failed to efficiently detect conformationally dependent antibodies. The quantity of correct peptides synthesized to the pins may also influence peptide-antibody interaction patterns. Geysen et al. (10) cautioned that a lack of binding cannot be taken as proof of the absence of antibody to that nominal sequence. We thus cannot conclude that the nonreactive peptides (215 of 367 hexapeptides) are not antigenic. Geysen et al. (9) previously reported that the probability of detecting antipeptide antibodies to a given site induced by native antigen is influenced by molecular stereochemistry, especially segmental mobility. Other studies (21, 33, 37) also suggest that the chemical nature of an amino acid sequence correlates with antigenicity. On the basis of a transmembrane protein, our data only had limited correlation with the probability of β-turn occurrence and no correlation with hydrophilicity or estimates of surface exposure (data not shown). The reason may be that the usual secondary structure prediction methods, which are designed for water-soluble proteins, are not as predictive for integral membrane proteins (25).

Among all regions in the B MOMP, we found that VD IV was immunodominant. We could further map epitopes in VD IV using rabbit polyclonal antibodies. High-resolution mapping confirmed that VD IV is antigenically complex, with variable topology and immunoaccessibility among serovars. Six continous but discrete linear antigenic sites were mapped; four of the six sites were immunodominant.

The antigenic complex within serovar B VD IV appear to contain a B-type-specific epitope (S1) in addition to known serogroup-specific (S2 and S4 to S6) and species-specific (S3) epitopes. S1 was mapped to VD IV by absorption of peptide 289-reactive antibodies with only serovar B-viable or fixed EBs. At the amino acid level, S1 does not share critical residues with adjacent sites (Fig. 3). Sequence substitutions in the S1 region are common among serovars, with 2 to 9 of the 10 amino acids substituted in this region (38). Serovar Ba is the only serovar identical to serovar B through this site. We infer that these amino acid substitutions are critical for antigenicity and abrogated binding (10). Our antigenicity data together with the sequence data suggest that S1 is an independent B serovar-specific epitope distinct from that previously mapped to VD II. These data are consistent with the previous report of Zhang et al. (39) that serovar B-specific MAbs recognized two distinct epitopes as determined by heat sensitivity and periodate susceptibility. Since S1 is both immunodominant and surface exposed (Fig. 3), we suggest that this B-type-specific determinant also contributes to the induction of serovar-specific immunity that has been noted in both animal models (34) and during human vaccine trials (12). Stephens et al. (29) reported that antipeptide antibodies raised by a VD II B-type-specific peptide poorly recognized the native epitope, and we failed to detect the VD II B-type-specific antibodies. Since S1 peptide-reactive antibodies could bind to native epitope on viable organisms, S1 may be more significant than VD II in terms of synthetic vaccine development. However, a MAb is needed to confirm the existence of S1, and we still do not know whether S1 antibodies contribute to the highest type-specific antibody titer in the antisera as detected in the microimmunofluorescence assay.

Four subspecies antigenic sites (S2, S4, S5, and S6) were

also identified in VD IV; S2 and S6 were immunodominant. The critical residues for S2 antibody binding appeared to be X-D-X^1-TTLN, with X and X^1 representing amino acid substitutions. Although the antibodies binding to S2 can be significantly absorbed by serovars A, C, H, and I, this was clearly less than that which was absorbed by serovars B, D, E, F, K, and L2. S2 was only surface exposed on serovars B, D, E, F, K, and L2. Amino acid sequence homology among various serovars supports the finding of broad antigenic specificity of the S2 determinant (38).

S4 and S5 were immunorecessive regions (Fig. 1) and displayed a lower affinity for antibodies. They may represent a smaller component in a discontinuous epitope. S6, like S1, is immunodominant and of narrow antigenic specificity (B, E, and L2). A comparison of sequences shows that S6 in L2 and B has identical amino acid sequences and is quite different from serovars A and C, which supports our antigenic mapping data. Baehr et al. (2) also mapped a MAb L21-5 to S6, confirming the existence of this epitope.

We paid particular attention to S3, since this is a highly conserved epitope among *C. trachomatis* serovars. Our antigenic mapping data are very consistent with sequence comparison data and previous MAb mapping (2, 29). Antibodies binding to eight residues (TLNPTIAG) in the sequence-invariable domain within VD IV could be completely absorbed by acetone-fixed EBs from all 12 serovars studied. Although this region is hydrophobic and has a low probability of topographic protrusion (data not shown), the scanning data showed that S3 was immunodominant among rabbits. Recently, Amit et al. (1) found that in a Fab-lysozome complex, 11 of 17 antibody contact residues were hydrophobic, with only 12 hydrogen bonds and no electrostatic interactions, indicating that hydrophobic forces dominated in the antibody-antigen interaction probably by efficiently excluding solvent. Thus the hydrophobicity of the S3 residue might provide potential antibody-binding ability. The immunodominance of S3-VD IV observed in rabbits is probably different from that observed in mice, since the generation of species-reactive MAbs in mice is a rare event (3, 20, 26, 28). Whether S3 is immunodominant or immunorecessive in humans is unknown at this time. From the absorption data using native EBs, we concluded that S3 is surface exposed on 7 of the 12 serovars examined (A, D, E, F, K, L2, and L3) but not immunoaccessible on serovars B, C, H, I, and J. We did not test serovars Ba, G, or L_1.

Although the S3 sequence is conserved, its immunoaccessibility in native EBs is very different among different serovars. Polyclonal rabbit antibodies bound to S3 with critical residues of NPTI, which is different from previous mapping data using MAbs (2, 6, 24). Different antibodies to the same nominal region may recognize the same epitope differently because of slightly different critical residue requirements. This may provide an explanation for the varied observations concerning the immunoaccessibility of the species-specific antigenic determinant among different chlamydial serovars, since different monoclonal antibodies were used.

Peterson et al. (24) characterized a species-specific MAb E-4 and found that it can neutralize the infectivity of B, E, D, L2, K, and L3 but not H, J, or C in vitro. The broad serovar reactivity of this MAb was quite consistent with that of the surface exposure of this epitope on native organisms as assayed by dot blot. We mapped this MAb to the S3 region with critical residues of LNPTI (data not shown). We therefore conclude that S3 is a protective determinant as long as it is immunoaccessible to corresponding antibodies.

Since our original purpose in mapping B-cell antigenic determinants on the MOMP was to identify immunoaccessible epitopes, we think S3 is certainly a candidate; however, since S3 is not surface exposed on all serovars, other sites also need to be considered. A continuous epitope from S1 to S4 (residues 289 to 310) might be useful, since one or more epitopes are exposed on eight different serovars (A, B, D, E, F, K, L2, and L3). To encompass the C complex organisms (serovars C, J, H, and I), a separate analysis will be undertaken by using a similar strategy to define peptides with similar immunologic characteristics.

ACKNOWLEDGMENT

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